SYNTHESIS AND TESTING OF POLYMERS SUSCEPTIBLE TO HYDROLYSIS BY PROTEOLYTIC ENZYMES

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HYDROLYSIS	DEVELOPMENT	POLYMERS	MOLECULAR WEIGHT
ENZYMES	SYNTHESIS	EXPOSURE(GENERAL)	MOLECULAR WEIGHT DISTRIBUTION
MACROMOLECULES	BIODEGRADABILITY	POLYAMIDE PLASTICS	WEIGHT REDUCTION
TESTING	BIODETERIORATION	POLYESTER FIBERS	REDUCTION
ESTERS	LINKAGES	RESIDUAL	WEIGHT

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Certain specific enzymes are known to cause hydrolysis of amide and ester bonds in naturally occurring macromolecules. The present research is directed toward synthesis, testing, and development of synthetic macromolecules containing biodegradable linkages found in natural polymers; the biodegradation of the polymer is measured after exposure to the specific enzymes effective for the type of bond that has been introduced. Emphasis has been placed in this work on substituted polyamides and polyesters. Degradation was followed by

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appearance of the known cleavage product, i.e., amino and carboxyl groups, by molecular weight reduction of the residual solids, and by weight loss. Additional fungal growth studies were conducted on polycaprolactone.

Materials under study are benzylated nylons, amino acid-containing model compounds, polycaprolactone, polymers containing α-hydroxy acids, and modified gelatin.

We have found the amide linkages in a designed diurea-diester model compound to be hydrolyzed in the presence of the enzymes elastase and papain. Chymotrypsin removes the end ester groups. This diurea-diester compound has been converted into a polymerizable monomer by changing the end groups to hydroxyl, and further tests are in progress. Polycaprolactone (MW 15,000) was degraded by Rhizopus chinensis acid protease enzyme and supports growth of two Aspergillus fungi. Degradation occurs via hydrolysis of the ester linkage. We are now copolymerizing caprolactone with other monomers to obtain a more desirable product property mix.

There are several other materials in various stages of characterization which we believe hold promise, but sufficient results are not yet available.

FOREWORD

The investigation was begun in 1973 under the US Army Natick Laboratories' Pollution Abatement Program. The latter was established as a mission assignment from the US Army Material Command under the general guidance of Public Law 91-190 (1969) and Executive Order 11507 which established a national pollution abatement policy. The present effort was based on an unsolicited proposal from the University of Connecticut to investigate new or modified nylons and polyesters which might be susceptible to enzymatic degradation. The intent in funding the work was not only to obtain potentially biodegradable new polymers but also to provide financial support to students and thus help provide this country with manpower trained in the pollution abatement field. This report is the final report of the first year of effort and marks the completion of Grant No. DAAG-17-73-G0002. Presently the work is continuing under Contract No. DAAK-03-74-C-0231 and is funded under project 1T762720D048-02-002 titled "Environmental Quality Research and Development - Natick". Dr. Richard N. MacNair and Mr. John T. Stapler of the Clothing and Personal Life Support Equipment Laboratory acted as the Project Officer and Alternate Project Officer, respectively.

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I. INTRODUCTION

A. Aims and Objectives

Very few studies of polymer biodegradability have been reported, and these have almost always involved burial of a polymer specimen, followed by measurement of weight loss and mechanical properties after a given period of time. Since such tests have shown that plastics and fibers of commercial interest degrade extremely slowly at best, and since such tests provide no information as to desirable polymer (or soil) modifications, an approach in which specific linkages in the polymer backbone are modified in such a manner as to be susceptible to hydrolysis by certain known enzymes appeared advantageous to us.

The overall objective is to develop synthetic biodegradable materials with desirable physical and mechanical properties for which the mechanism of degradation is known and, if possible, controllable.

The starting point for this research was the premise that since natural polymers such as proteins are readily broken up into small segments by certain known enzymes, controlled modification of polyamides to make them similar to the natural materials in certain critical respects should result in biodegradable synthetic polyamides. Initially benzyl groups were placed on one of the diacid α carbons with the objective of making the

$$-HN(CH_2)_nNHCOCH(CH_2)_nCO-$$
$$\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad CH_2Ph$$

amide linkage more phenylalanine-like and recognizable to the enzymes. As the work progressed it became apparent that not only the α carbon substituent but also the groupings on either side of the amide linkage are important, and to

program was expanded to vary these groupings in a systematic manner. The reported degradability of polycaprolactone in the presence of fungi[1] also led us to investigate the biodegradability of polycaprolactone and other polyesters in the presence of esterases. Several polymers containing mixed amide and ester linkages have also been studied.

B. Report Organization

Before measurements of biodegradability of the various polymers could proceed, it was necessary to develop both meaningful procedures for exposure of the samples to the enzymes and means of characterization of the solid and liquid products remaining after the exposure. This becomes difficult because the enzymes possess many of the same structural characteristics as the synthetic polymer samples. The results in Section III, "Synthesis of Materials and Degradation Testing", must therefore be viewed in the perspective of the test methods and conditions that were used, which are described in Section II, "Methods Development". For example, it is shown that weight loss as a measure of degradation is subject to considerable error.

The various synthetic polymers in Section III are grouped in four categories:

1. Benzylated nylons
2. Model compounds containing α amino acids
3. Polycaprolactone
4. Polymers containing α hydroxy acids

II. METHODS DEVELOPMENT

A. Exposure of Materials to Enzymes

After the synthesis and purification of a new material, a degradation study was initiated as follows: Into 16 ml corrosion resistant borosilicate glass vials were added 100-200 mg of powdered polymer (depending on the availability

of the polymer). Samples were run in duplicate for each condition. The vials for the samples to be subjected to enzyme attack were filled with 14 ml of 0.2M buffer solution at the appropriate pH for the enzyme used. Azide was added to prevent microbial contamination. Then 1 ml of enzyme solution at about 1 mg/ml concentration was added. Two controls were used for each sample: The first contained the polymer plus the buffer (15 ml) and was used to determine the amount of polymer leached or hydrolyzed by the buffer alone. The second contained buffer (14 ml) and enzyme solution (1 ml) to serve as a blank for the amine end-group determination on the supernatant liquor of the treated sample.

The sample plus controls were placed on a shaker table at ambient temperature for six to ten days. During this period, fresh enzyme was added to the sample and the enzyme-buffer control every 24 or 48 hours. One ml was withdrawn and saved while 1 ml of the enzyme solution at the original concentration was added.

At the end of the last day of reaction, the solids (if present) were filtered in the predried tared sintered glass or cellulose filters. The filtrate was saved and the remaining solid washed with at least 50 ml of distilled water. The solids were then air dried for 24 hours, then oven dried for 2 hrs at 60°C. The filters and solids were then weighed and the weight loss for the sample determined. Solids were kept for viscosity measurement. The filtrates (from both the sample and the material removed during enzyme replenishment) were analyzed for amine end groups using either a colorimetric ninhydrin assay or a titrimetric assay with HCl.

6. Enzyme Assays

It was necessary to monitor enzyme activity as a function of time and temperature to determine how frequently enzyme must be replinished during the

10-day runs. Standard methods were used to assay activities using known substrates. We also verified that the enzymes were not inactivated by physical adsorption to our benzylated nylon samples. A 6 day test with thermolysin, chymotrypsin, and subtilisin showed that the mere presence of the polymer caused little loss in enzyme activity over the controls without polymer. Similar results were obtained with the sulfonated benzyl-nylon using chymotrypsin and subtilisin.

C. Analytical Procedures for Measuring Degradation

1. Weight Loss

By weighing the solid polymer before and after exposure to enzyme, a simple measure of degradation is possible. The method assumes that all degradation fragments are soluble, regardless of molecular weight. We have found, however, that the method is difficult to use because of several problems: a) Soluble charged fragments may adsorb to cellulose or glass, thereby interfering with both weight loss and end group detection methods. b) Filtration rates on filter paper or on small (3 ml) glass frits are very slow, because fine pore filters must be used. c) Centrifugation avoids problems a and b, and it has been used to separate solids. However, fine particles often remain suspended because of surface or density effects. It is difficult to remove all the pellet from the tube for weighing (A 15 ml centrifuge tube is too heavy for tareing against the 100 mg. sample). d) It is difficult to detect degradation below 10% in small 100 mg. samples, and therefore the weight-loss method is less reliable than amino end group analysis or viscosity measurement. For the above reasons we feel that weight loss provides only a rough and sometimes inaccurate indication of degradation, and that only large consistent differences between the samples and controls have meaning.

2. Amino End-Group Analysis

The NH_2 groups generated by enzyme cleavage of amide NH-CO bonds can be detected in filtrates or even in the remaining solids if a suitable solvent is available. Two procedures have been used: titration with acid and colorimetric analysis with ninhydrin reagent.

a) Acid Titration

Solid fragments left after enzyme degradation of a benzylated 6,6 nylon were solubilized in 70% (w/w) phenol-methanol and were titrated with 0.05N HCl in the presence of thymol blue indicator (pH 1.2-2.8). The amount of titrant per gram solids was converted to number of end groups per gram, and was compared with the value obtained for the original sample.

b) Ninhydrin Analysis

This is a very sensitive assay and can detect primary amino groups in the μM range. For this reason, it was necessary to run control measurements on the buffer-enzyme and buffer-polymer mixtures. It was also necessary to deaminate the distilled water, for which a Durrum DC-30 cation exchange column was used.

The following analytical procedure was used for appearance or disappearance of primary amino groups: Two gm of recrystallized ninhydrin was added to 50 ml of 2-methoxyethanol. 0.08 gm of tin chloride dihydrate was added to 50 ml of 0.2M citrate buffer at pH 5.0. Just prior to use, the ninhydrin solution and the tin chloride solution were mixed. Due to the instability of this solution it was made up fresh for every assay. A solution of 50% (V/V) i-propanol in water was prepared as a diluent.

One ml of the ninhydrin solution was then added to a test tube. To this was added 0.10 ml of the solution to be tested and the resulting mixture was shaken and covered with aluminum foil caps. The tubes were then immersed in boiling water for 20 minutes. Care must be taken to suspend the tubes so that

even heating is maintained. After 20 minutes the tubes were immersed in cold water and 5 ml of diluent were added.

The optical density of the samples was read at 570 mμ on a Beckman spectrophotometer against a blank of distilled water treated the same way as the sample. A plot was made of the optical density of the standards versus concentration in the 0.5 μM range. Samples should be within 5% of each other.

3. Intrinsic Viscosity

Polymer degradation has been followed by viscosity changes, which are related to molecular weight by the Mark-Houwink equation: $\eta = KM^a$. The benzylated nylon samples were dissolved in 90% formic acid for the measurement, and the constants for nylon 6,6 were used to obtain molecular weights. For caprolactone samples, benzene was used as a solvent.

An automatic Fica viscometer has been used for routine viscosity measurements. This instrument has a photocell to sense meniscus movement and to initiate a timer accurate to milliseconds. It is equipped with a dilution device (±0.01ml).

To utilize this accuracy, computer programs have been written to analyze the data. One program is used on batch time on the IBM 360/65 computer. By adding the viscometry data to the program deck, a complete analysis is available in minutes. This program does a least-squares curve fit of the Huggins, Kraemer, and Maron-Reznik equations. It gives the viscosity from each equation and all the constants.

A CPS (terminal time) program is in the PSC library of our IBM 360/65 computer. This program is rapid (5 minutes) and inexpensive ($0.22). The program gives the viscosity as determined by a least-squares curve fit of the Maron-Reznik equation.

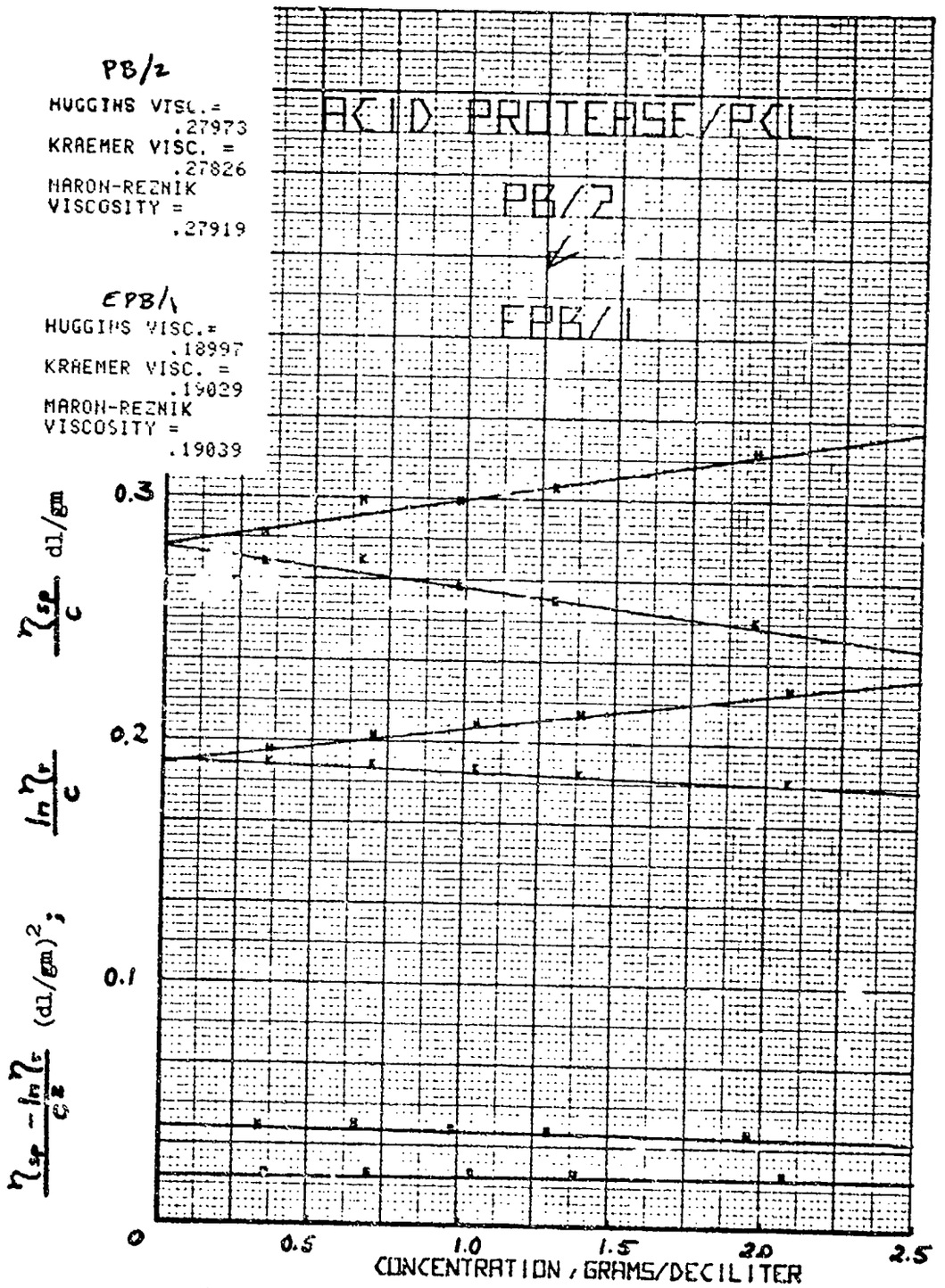


Figure 1: Viscometry Plots of Polycaprolactone. Polycaprolactone (PCL) was reacted with acid protease for six days. EPB/1 is an enzyme-polymer-buffer sample. PB/2 is a polymer-buffer sample. Viscosities were run using benzene as a solvent. The plots are: Huggins equation-H; Kraemer equation-K; Maron-Reznik equation-M. The higher line of each pair is the PB/2 data. The lower lines are from EPB/1. The intercept of the lines (concentration zero) represents the viscosity and is related to molecular weight.

A program has also been written for the Hewlett-Packard 9820 calculator-plotter. This program plots the data points, fits a curve to these points, and gives the viscosity. It does this for all three equations (see Figure 1). Bad data points can be deleted, if necessary, and the plots can be redrawn. These computer programs together with the Fica Viscometer have made our polymer molecular weight analysis quick, simple, and accurate. An error analysis of the data will soon be added to the programs.

4. Dye Release

The release of entrapped dye molecules during degradation of dyed materials can, in principle, be used to measure the rate of breakdown. Test experiments were run on nylon 6 dyed with edicol red. The enzyme used was elastase at pH 9. Final results were inconclusive because the dye easily leached out in the basic solutions. Later trials indicated congo-red in tris buffer at pH 8.4 is a better dye for basic media. After 6 days of enzyme treatment, negligible degradation of nylon 6 was found. Further experiments using this procedure have not been tried. The method should, however, be useful for polymers devoid of amino linkages, for which the ninhydrin end-group analysis could not be used, and especially when small amounts of available material would not be sufficient for weight loss analysis.

5. Fungal Growth

a) Agar Plates

A standard technique for testing fungal growth on polymers is provided in ASTM method D1924-61T. The procedure requires three plates: polymer, filter paper, and a control, all using non-nutrient agar devoid of utilization carbon. The filter paper control should show heavy growth indicating fungal viability, while the blank control should show no growth on the agar medium itself. We found, however, that fungi were able to grow lightly on the non-nutrient agar blank control. This is due to agar decomposition which commonly occurs

during autoclaving. (See polycaprolactone results.)

b) Spore Suspensions

To further confirm that these molds are assimilating the polymer and not just the residual sugars of the agar, another experiment was initiated. Polymer strips, cut from polymer plates pressed by ram compression, were inserted half way into a spore suspension. No agar was used, but basal salts were in the suspension. See Figure 2.

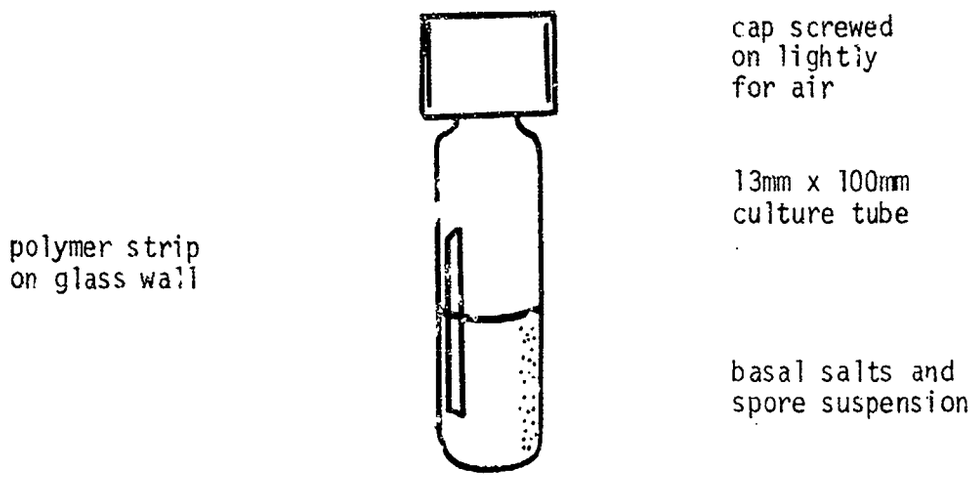


Figure 2. Position of Polymer Test Strip in Basal Salts for Fungal Growth Studies.

Two controls were used: A piece of sterile filter paper was used for a spore viability control, and a polymer strip in basal salts was used to show whether any degradation occurred due to the salt water. Samples were done in duplicate at 30° ± 2°C. (For results, see caprolactone section).

III. SYNTHESIS OF MATERIALS AND DEGRADATION TESTING

Efforts have been directed toward the syntheses of polymers and model compounds that might be degradable by chain-cleaving endopeptidases and esterases. Compounds containing amide, ester, urea, and urethane linkages

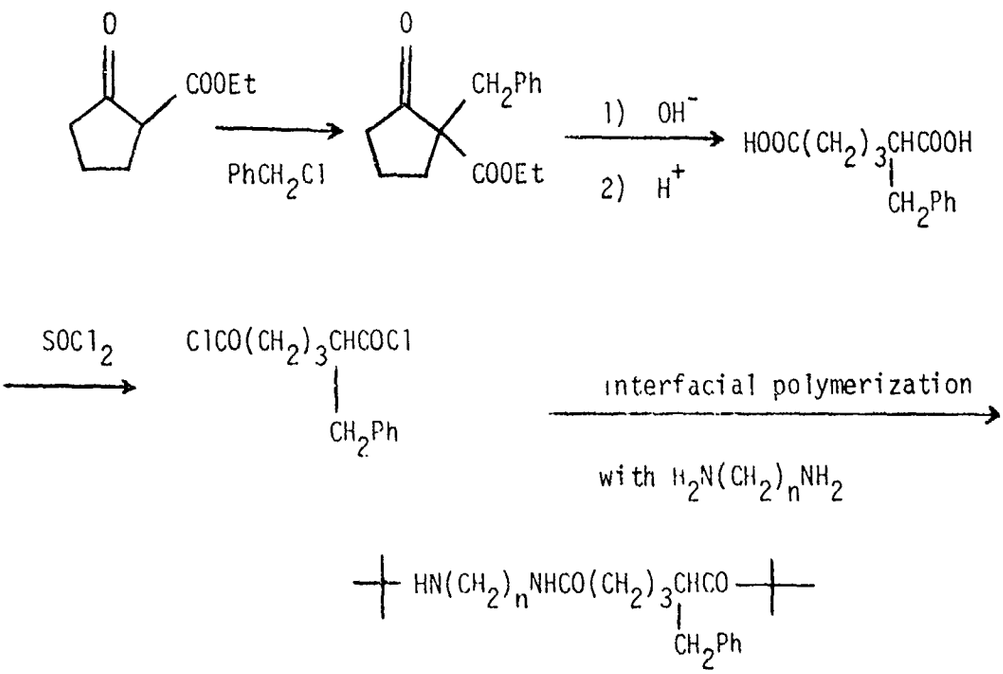
have been prepared. The preparations and testing results are described in the following sections.

A. Benzylated Nylons

Our initial testing of nylon-6 and nylon-6,6 showed that these polymers resist enzyme hydrolysis. Since several enzymes (chymotrypsin and pepsin, for examples) are specific in cleaving peptide linkages adjacent to aromatic groups we decided to synthesize α-benzylated nylons, anticipating that the introduction of the α-benzyl group would make the nylons more susceptible to enzyme cleavage.

Nylon- ,6 αBz and nylon-6, 3 αBz were synthesized according to Scheme 1 and Scheme 2, respectively.

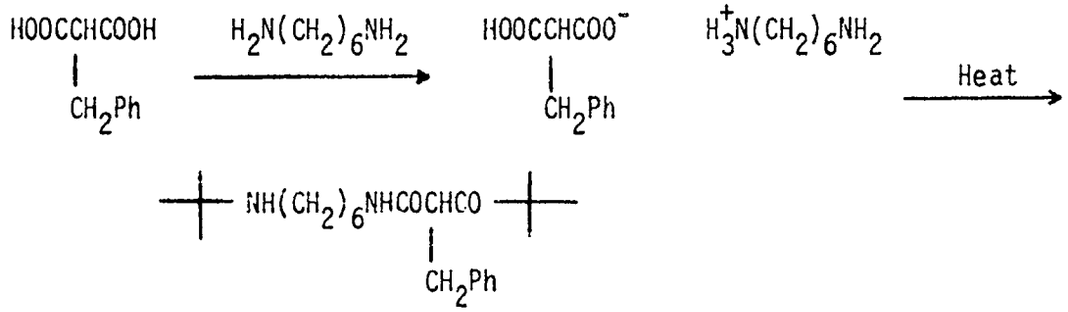
Scheme 1. Syntheses of Nylon-n, 6αBz



Nylon-n,6αBz

The nylon-n,6αBz polymers thus synthesized have Mn (by amino end group titrations) in the order of 2×10^3 with softening points: 2,6αBz, 145°; 4,6αBz, 140°; and 8,6αBz, 105°.

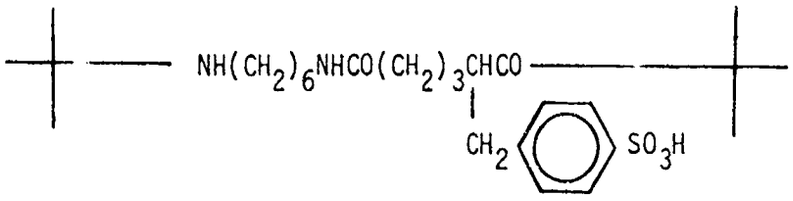
Scheme 2. Synthesis of Nylon-6,3αBz



Nylon-6,3αBz

Nylon-6,3αBz thus prepared has molecular weights ranging from 10^3 to 10^4, dependent upon the polymerization conditions. Since nylon-6,3αBz contains two "phenylalanine-like" amide linkages quite different from that of the nylon-n, 6αBz series, it might have different degradability compared to that of the nylon-n,6αBz series.

To study the effect of the water solubility of the nylons on the degradability we next sulfonated nylon-6,6αBz to give the water soluble nylon-6,6αBzSO_3H.



Nylon-6,6αBzSO_3H

It was recognized that the large and polar SO_3H group might itself hinder enzymatic attack on the backbone, and an effort was started to replace the

SO_3H group with an OH group. This effort was abandoned, however, when it was later found that the very similar polytyrosine showed no evidence of backbone cleavage when treated with chymotrypsin.

The above modified nylons were next exposed to various enzymes. The results are listed in Table I. Initial experiments with nylon-n,6αBz indicated 5-15% weight loss after exposure to chymotrypsin with the values obtained at low pH. Measurement of the appearance of new amino end groups (by the ninhydrin method, described in the Test Development section) showed no increase, however. It was concluded that no appreciable chain cleavage was occurring.

A sample of nylon-6,3αBz after exposure to imidazole buffered chymotrypsin showed relatively high weight loss (42% in buffer and 47% in buffered chymotrypsin) as compared with the results of that of the nylon-n,6αBz series. We are continuing on the study on nylon-6,3αBz.

Thermolysin, a relatively heat-stable enzyme, was used at three temperatures in hope that the nylon structure would loosen up at higher temperatures, thus allowing better approach of enzyme to individual polymer chains. In fact, however, a greater weight loss was seen at lower temperature.

A solubilizing agent, SDS, was next used with thermolysin to swell the polymer material. At 25°C the molecular weight of the remaining solid actually increased. This may mean that smaller Mw components were leached out of the sample during the soaking experiment. Leaching would occur in both control and sample, so no net weight loss is expected.

Table 1. Results of Enzyme Degradation Studies on Nylons[a]

Polymer	Enzyme used 0.1-0.5 mg/ml	Buffer, pH (temp,°C)	Wt. Loss,%[b]	Amino end group[c,e,g] increase,%
Nylon-6	chymotrypsin	acetate, 3-7 (rt)	< 1	
"	"	phthalate, 3-7 (rt)	< 2	
"	pepsin	acetate, 3-7 (rt)	< 2	
"	"	phthalate, 3-7 (rt)	< 2	
"	elastase	tris, 9 (rt)	0[d]	
Nylon-6,6	chymotrypsin	acetate, 3-7 (rt)	< 2	< 1[e]
"	"	phthalate, 3-7 (rt)	5.6 pH 7	< 1[e]
Nylon-6,6αBz	"	imidazole, 7.5 (rt)	5.7 ± 1	< 1[c,e]
"	"	phthalate, 5.8 (rt)	0.3	< 1[c,e]
"	"	HCl, 3.0	15	
"	subtilisin	phosphate, 7.5 (rt)	< 1	
"	thermolysin	tris, 8.0 (25°)	16 ± 5	
"	"	tris, 8.0 (37°)	< 1	
"	"	" (47°)	< 1	
"	thermolysin with SDS	" (25°)	0.6	< 1[c]
"	"	" (47°)	5.0	< 1[c]
Nylon-6,6 αBzSO$_3$H	chymotrypsin	imidazole, 7.6 (rt)	-	< 1[c]
"	subtilisin	phosphate, 7.5 (rt)	-	< 1[c]
Nylon-2,6 αBz	chymotrypsin	HCl, 3.0 (rt)	6 ± 3	
"	"	phthalate, 5.8 (rt)	< 1	
"	"	imidazole, 7.5 (rt)	2 ± 1	
Nylon-4,6 αBz	"	HCl, 3.0 (rt)	-	
"	"	phthalate, 5.8 (rt)	8	
"	"	imidazole, 7.5	< 1	
Nylon-8,6 αBz	"	HCl, 3.0 (rt)	7	
Nylon-8,6 αBz	"	imidazole, 7.0	1	
Nylon-8,6 αBz	"	phthalate, 5.8 (rt)	< 1	
Nylon-6,3 αBz	"	imidazole, 7.0	5 ± 0.5[f]	

Notes

a. Data are averages from duplicates or triplicates.

b. Wt. loss differences from exposure to buffered enzyme solution compared with that from exposure to buffer only.

c. Ninhydrin analysis.

d. No degradation as indicated by dye release method.

e. HCl titration.

f. 47% after exposure to buffered chymotrypsin and 42% after exposure to imidazole buffer only.

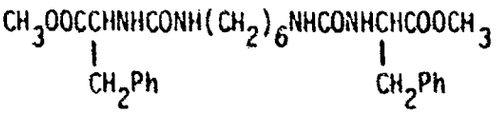
g. This result is expressed as a percentage of the possible new groups which could have appeared if all the CONH bonds in the polymer were hydrolyzed.

B. α-Amino Acid Containing Model Compounds

It has been reported that copolymers of amino- and hydroxyacids are easily hydroloyzed[2]. We decided to synthesize copolymers containing amide, ester, urea, and urethane units, especially those with α-amino acid residues incorporated in the polymer main chain. In order to investigate the problem in a systematic manner we first synthesized several model compounds and studied their enzyme degradabilities.

1. Model Compounds Containing Phenylalanine Residues

The diester-diurea model compound, L,L-C_1-EsPheUa-C_6-UaPheEs-C_1, was prepared in 89-94% yield by the reaction of L-phenylalanine methyl ester with 1,6-diisocyanatohexane. Similarly, the



C_1 - EsPheUa-C_6-UaPheEs-C_1

D,L - isomer was obtained in 90% yield. Preliminary enzyme degradation studies have yielded promising results (Table 2).

Table 2. Results of Enzyme Degradation Studies on C_1-EsPheUa-C_6-UaPheEs-C_1[a]. Diester-Diurea model, R = benzyl.

$CH_3OOCCHRNHCONH-(CH_2)_6NHCONHCHRCOOCH_3$

Isomer	Enzyme Used 0.1 mg/ml	Buffer,pH	Wt. Loss, %[b]	Ninhydrin Amino End Group Increase[c]
DL	chymotrypsin	imidazole, 7.8	27	0
LL	chymotrypsin	imidazole, 7.8	79	-
DL	elastase	tris, 8.8	-	<1
LL	elastase	tris, 8.8	12	7
DL	papain	PO_4,6.5	24.7	6
LL	papain	$PO_4$6.5	1.5	8
DL	acid protease	gly. HCl,3.1	0	error
LL	acid protease	phthalate,3.1	1	<1
LL	pepsin	HCl 2.2	<1	-

Notes

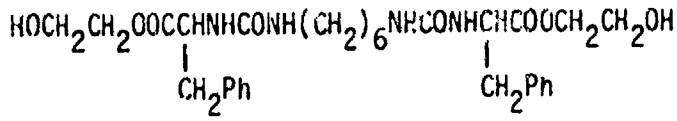
The weight loss data show that chymotrypsin cleaves the outer ester bonds of the model compound, preferentially the LL isomer, as expected. A gas chromatographic analysis is underway to confirm the existence of methanol in the filtrate. No free NH_2 groups were found. Elastase and papain appear to be attacking one of the ^{2} amide bonds. Whether they cleave the ester bond also will be shown by the methanol analysis. The two acid active enzymes appear to have no effect on the model compound.

a. Data are averages for duplicates or triplicates.

b. Wt. loss differences from exposure to buffered enzyme solution compared with that from exposure to buffer only.

c. This result is expressed as a percentage of the possible new groups which could have appeared if all the CONH bonds in the polymer were hydrolyzed.

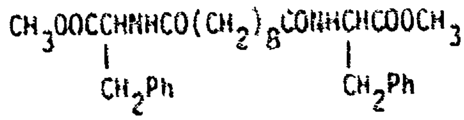
Using phenylalanine monoglycol ester, we have now prepared the monomer-model compound L,L-HOC_2-EsPheUa-C_6-UaPheEs-C_2OH.



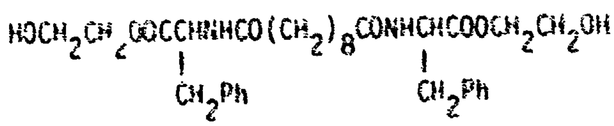
HOC_2-EsPheUa-C_6-UaPheEs-C_2OH

Enzyme degradation studies are in progess. If the results are positive, polymerizations of this biodegradable monomer with diacids will give copolymers containing ester and urea linkages, whereas polymerizations of this monomer with diisocyanates will give polymers containing ester, urea, and urethane linkages.

The diamide-diester model compound, C_1-EsPheA-C_8-APheEs-C_1, was prepared from the reaction of phenylalanine methyl ester with sebacoyl chloride. It also will be subjected to enzyme degradation tests. The corresponding monomer HOC_2-EsPheA-C_8-APheEs-C_2OH and polymers derived from the dihydroxymonomer will be synthesized if the enzyme degradation studies on the model compound turn out to be positive.



C_1-EsPheA-C_8-APheEs-C_1



HOC_2-EsPheA-C_8-APheEs-C_2OH

2. Model Compounds Containing Glycine Residues:

In order to compare the degradabilities of phenylalanine-containing materials with that of glycine-containing materials we plan to synthesize and

study the degradabilities of glycine-containing model compounds, monomers, and polymers structurally similar to those phenylalanine-containing model compounds, monomers, and polymers mentioned above, except glycine will be used in place of phenylalanine. We have now prepared the model compound C_2-EsGlyUa-C_6-UaGlyEs-C_2:

$$C_2H_5OOCCH_2NHCONH(CH_2)_6NHCONHCH_2COOC_2H_5$$

C. Polycaprolactone

1. Enzyme Studies

This commercial polymer (MW 14,000) was a good subject for enzymatic degradation study because Union Carbide had reported that it supported fungal growth in lab cultures and in ground burial tests[1]. Our enzyme study shows significant molecular weight decrease when the material is directly exposed to the acid protease from Rhizopus chinensis, Table 3. Weight loss may not have been detected for the several reasons discussed in Methods Development. The remaining four esterases appear to be less effective, though work with elastase is continuing.

Table 3. Results of Degradation Studies on Polycaprolactone (polyester-6)[a]

Enzyme 0.1 mg/ml	Buffer, pH	Wt. loss, %[b]	Viscosity $\bar{M}_w$ decrease %
acid protease	phthalate, 3.3	1	32[c]
papain	imidazole, 7.0	<1	< 2
protease VI[d]	imidazole, 7.5	3	< 1
subtilisin	phosphate, 6,7,8	0	-
elastase	tris, 8.8	in progress	

a. Data are averages from duplicates or triplicates.

b. Since the initial $\bar{M}_n$ = 14,000, only very extensive cleavage to give low molecular weight products will be reflected in wt. loss measurements.

c. $\bar{M}_n$ from 13,000 to 10,000.

d. Strentomyces griseus, Sigma Chemical Co.

2. Fungal Growth Studies

Union carbide workers combined four fungi in their cultures[1]. We have applied each separately to polycaprolactone to help establish which enzyme or class of enzymes is actually causing the degradation. A survey of the literature has shown which enzymes are predominately formed by each fungus.

Our results are shown in the photographs. Four cultures were first grown on non-nutrient agar (see Methods Development for details): Aspergillus niger, A. flavus, Penicillium funiculosum, and Chaetomium globosum. Light to moderate growth was observed in all cases, especially with the two Aspergillus strains. Growth was localized around edges and cracks of the polymer, rather than on surfaces as indicated in Figure 3. This could be due to an inhibitory coat of Al_2O_3 deposited on the polymer surface while pressing the material with aluminum foil. This will be checked by using solution cast polymer samples.

Culture tube spore suspensions have been used to confirm that these fungi are not assimilating the residual sugars in the agar. The four tubes are shown in Figure 4 with two controls. After eight days at 30°C all were growing on the polymer strip except C. globosum. Of the many enzymes produced by Aspergillus, rennin is the predominant esterase and it will therefore be used in future work.

D. Polymers Containing α-Hydroxy Acids

Esters of hydroxy acids that can undergo lactone formation are known to be easily hydrolyzed. The rate enhancement of those hydroxy-esters over analogous esters without the hydroxy groups (10^3 - 10^6 fold rate increases were reported) has been attributed to self-catalyzed lactone formation[3].

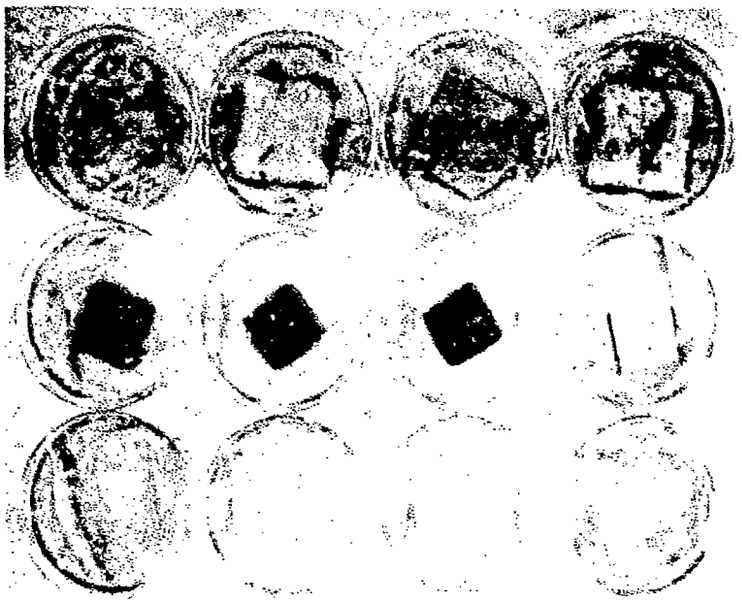


Figure 3. Fungal Growth Experiment with PCL using Non-nutrient Agar. The 4 columns are (l. to r.): Aspergillus flavus, Chaetomium globosum, Penicillium funiculosum, and A. niger. The 3 rows are (t. to b.): polymer samples (PCL), filter paper viability control, and non-nutrient agar control.

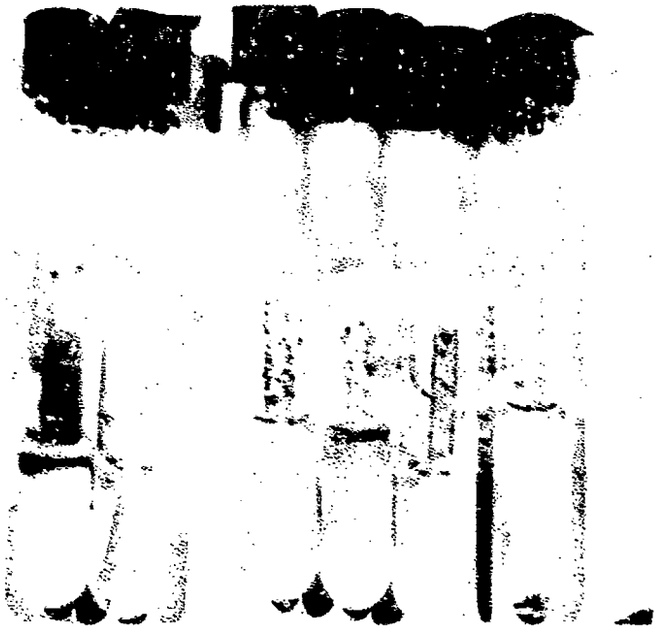
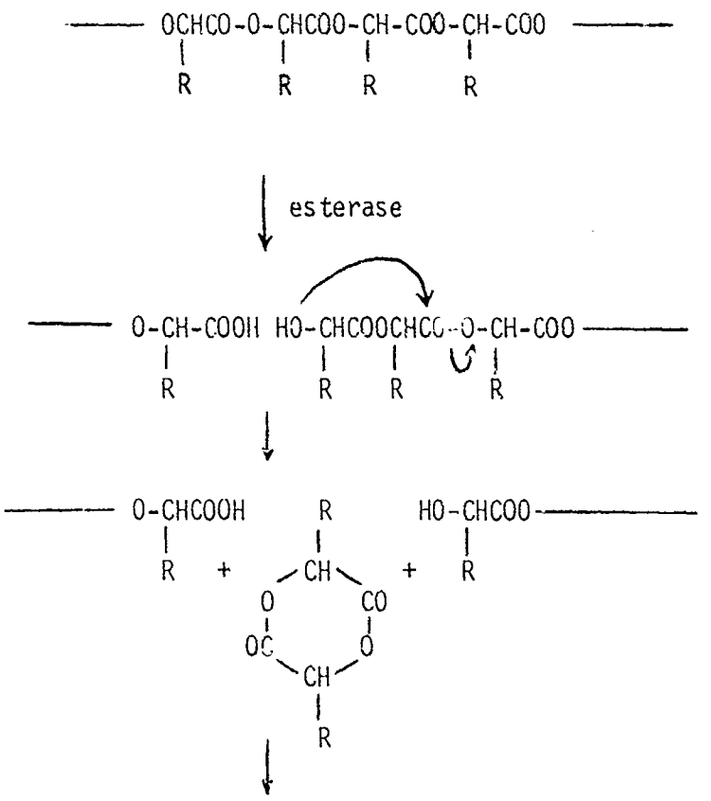


Figure 4. Culture Tube Strip Growth Experiment. l. to r.: spore viability control with filter paper (P. funiculosum), an erosional control without spores (PCL in basal salts), A. niger on PCL, A. flavus on PCL, P. funiculosum on PCL, and C. globosum on PCL.

It seems to us that polyesters derived from α-hydroxyacids might be very degradable since the six-member dilactone formation could be very favorable once the polyester is cleaved by esterase.

Scheme 3.



This self catalyzed reaction will result in depolymerization. We have synthesized poly(mandelic acid), (polyester 2-Ph), and carried out preliminary hydrolysis studies. Results to date are listed in Table 4.

Table 4. Results of Degradation Studies on Polyester-2Ph (Poly mandelate)[a]

Enzyme used 0.1 mg/ml	Buffer, pH	Wt. loss, %[b]
Subtilisin	phosphate, 6,7,8	3 at pH 8
Axion detergent[c]	phosphate, 8,10	40
Chymotrypsin	imidazole, 7.5	<1

a. Data are averages from duplicates or triplicates.

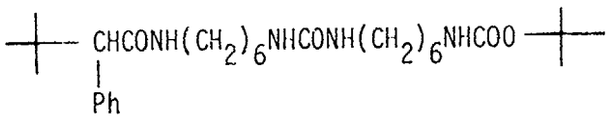
b. Wt. loss difference from exposure to buffered enzyme solution compared with that from exposure to buffer only.

c. It is believed that Axion contains subtilisin and surface active agents. The large difference between Axion and buffered subtilisin might be due to surface active agents that will loosen up the polymer.

Polymers derived from α-hydroxyacids and 1,6-diisocyanatohexane

Since polymers containing aliphatic ester main chains are generally low melting and have wax-like physical properties, we decided to prepare copolymers containing ester as well as amide, urea, and urethane linkages in the main chain. The introduction of H-bond forming linkages should increase the melting points of polyester and perhaps also the fiber forming properties of the polymer.

The reaction of mandelic acid with 1,6-diisocyanatohexane was used to give a polymer containing amide, urea, and urethane linkages poly(C_1Ph-A-C_6-Ua-C_6-Ue)



Poly (C_1Ph-A-C_6-Ua-C_6-Ue)

Nuclear magnetic resonance (NMR) analysis showed that the polymer contains one mandelic acid residue to every four 1,6-hexamethylene diamine residues.

In a similar manner the reaction of glycolic acid with 1,6-diisocyanatohexane gave poly (C_1-A-C_6-Ua-C_6-Ue).

$$\left\{ CH_2\text{-}CONH(CH_2)_6NHCONH(CH_2)_6NHCOO \right\}$$

Poly (C_1-A-C_6-Ua-C_6-Ue)

NMR analysis showed that this polymer contains one glycolic acid residue to every five 1,6-hexamethylene diamine residue. Enzyme degradation studies on these polymers are now in progress.

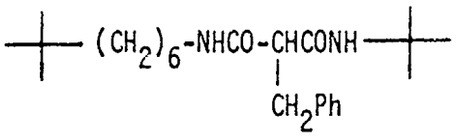
E. Exploratory Work on Modification of Gelatin:

Gelatin, a readily available water soluble protein mixture, contains around 4% of lysine and 8% of arginine residues. The second amino groups of lysine and arginine together with the amino end groups of the protein chains might be expected to react with suitable monomers such as diisocyanates and diacid chlorides to give water insoluble high molecular weight linear chain and/or crosslinked polymers, dependent on the extent of reaction. Polymers thus obtained should be degradable since they contain degradable protein segments. It is expected that those polymers might be spinable into fibers having wool or silk-like properties. We have very recently begun exploratory work in this area. Reaction between an aqueous solution of gelatin and 1,6-diisocyanatohexane in organic solvent gave a white, water-insoluble polymer. Characterization of this polymer is now in progress.

IV. CONCLUSIONS

A. Benzylated Nylons

Of the several benzylated nylons that were investigated, only the nylon 6,3 appears promising, and results on this polymer are only preliminary. Nylon 6,3 does have a unique structure:

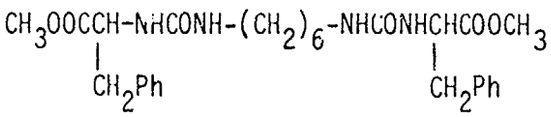


which may cause differences relative to higher homologs in which CH_2 sequences are present. Admittedly, measurements on some of the higher homologs were not extensive, due to the considerable time required, but the very low weight loss results were reproducible and were substantially the same for all cases.

Solubilization of benzylated nylon 6,6 via sulfonation in the para ring position did not help, and it was found that soluble polytyrosine was not degraded by suitable enzymes. This fact plus the observation that a crystalline, insoluble model compound (next section) showed good degradation rates leads us to believe that lack of mobility of chains in the solid state does not per se prevent degradation. The lack of decrease in molecular weight when a swelling agent (SDS) was used with the enzyme also supports this line of reasoning. Further work is needed, however.

B. Model Compounds

The diester-diurea model compound



shows markedly enhanced degradability in the presence of specific enzymes (elastase and papain) when the compound is in the L,L form. The D form shows no degradability, and D,L mixtures show degradability proportional to the percentage L form. Cleavage results in an increase in primary amino groups, showing that breakage occurs at the internal nitrogen atoms. Chymotrypsin, on the other hand, causes hydrolysis of the terminal ester groups. The compound with terminal OH groups rather than ester groups has now been prepared; we expect that it will also be degradable by papain and elastase, and can serve as a monomer for polymerization. Measurement of the degradability of the di-

hydroxy compound is in progress. A third compound in which the methyl ends shown above were replaced by ethyl, and also the non-benzylated compound, have been prepared. Tests are still underway, and thus all we can say is that the chymotrypsin does hydrolyze the terminal ester linkages but not the amide linkage. The ninhydrin analysis on samples exposed to papain and elastase will determine whether or not benzylation is necessary. We are also seeking to determine the necessary sequence of backbone atoms.

C. Polymers Containing Hydroxy Acids

Homopolymers derived from hydroxy acids (polycaprolactone and polymandelate) are degradable in some cases. Since these polymers do not have the desirable physical properties for applications as fibers (polymers containing aliphatic ester chains are generally wax-like, brittle, and low melting), we are now in the process of preparing copolymers containing ester, amide, urea, and urethane linkages, anticipating that the introduction of H-bond forming amide, urea, and urethane linkages into a polyester chain will improve the physical properties of the polyesters but retain the degradabilities.

D. Methods Development

New analytical methods were used for measuring biodegradability; samples were exposed to specific purified enzymes in a controlled environment, and generation of expected new end groups was measured by ninhydrin or titrametric analysis (for primary amino groups). Changes in polymer molecular weight and sample weight were also monitored. These techniques provided not only a measure of the damage to the polymer, but how and where this damage occurred along the molecular chain.

A new technique is also reported for fungal growth measurements on polymers, and some of the pitfalls of other methods are discussed.

V. REFERENCES

1. J. E. Potts, R. A. Clendining, W. B. Ackart, and W. D. Niegisch, "Polymers and Ecological Problems", J. Guillet, Editor, Plenum Press, New York, 1973, pp. 61-80.
2. German Pat. DOS 216215 Angew. Chem. Int'l. Ed., 12, 937 (1973).
3. B. Capon, S. T. McDowell, W. V. Raftery, J. Chem. Soc. Perkin II, 1118 (1973).